

Cardiopulmonary Support and Physiology

ONO-5046 attenuation of delayed motor neuron death and effect on the induction of brain-derived neurotrophic factor, phosphorylated extracellular signal-regulated kinase, and caspase3 after spinal cord ischemia in rabbits

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Objective: The mechanism of spinal cord injury is believed to be related to the vulnerability of spinal motor neuron cells to ischemia. The aim of this study was to investigate whether ONO-5046, a specific inhibitor of neutrophil elastase that can attenuate tissue or organ injury in various pathologic conditions, could protect against ischemic spinal cord damage.

Methods: After induction of spinal ischemia, ONO-5046 or vehicle was injected intravenously. Cell damage was analyzed by counting the number of motor neurons. To investigate the mechanism by which ONO-5046 prevents ischemic spinal cord damage, we observed the immunoreactivity of CPP32 (caspase3), brain-derived neurotrophic factor, and phosphorylated extracellular signal-regulated kinase.

Results: ONO-5046 eased the functional deficits and increased the number of motor neurons after ischemia. The induction of caspase3 was significantly reduced by ONO-5046 treatment. Furthermore, the expressions of brain-derived neurotrophic factor and phosphorylated extracellular signal-regulated kinase were prolonged.

Conclusion: ONO-5046 may protect motor neurons from ischemic injury by reducing caspase3 and prolonging the expressions of brain-derived neurotrophic factor and phosphorylated extracellular signal-regulated kinase. ONO-5046 may be a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury.

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Spinal cord injury after a successful operation on the thoracic aorta is a disastrous and unpredictable complication in human beings. In an attempt to prevent this complication, various methods of spinal cord protection have been suggested, including temporary shunts, partial bypass, and systemic or regional hypothermia. Regardless of the surgical technique or method of spinal cord protection used,¹ however, none of the methods developed to date can totally protect against the possible development of paraplegia. The reported prevalence of paraplegia ranges from 2.3% to 23% for operations on the thoracic aorta.² The mechanism of spinal cord injury during operations on the thoracic aorta is believed to be primarily related to direct tissue ischemia. Ischemia can occur when there is

Abbreviations and Acronyms

BDNF	= brain-derived neurotrophic factor
ERK	= extracellular signal-regulated kinase
MEK	= mitogen-activated protein kinase/ERK kinase
p-ERK	= phosphorylated extracellular signal-regulated kinase

permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of the spinal cord blood flow.³ For a rabbit spinal cord ischemia model, we have previously reported delayed and selective motor neuron death after transient ischemia, which was strongly associated with activated apoptotic signals of the caspase3 cascade.⁴

It has been demonstrated that there is significant upregulation of brain-derived growth factor (BDNF) in peri-ischemic regions after a noninjurious interval of middle cerebral artery occlusion in the rat.⁵ Furthermore, increased expression of BDNF evoked by spreading cortical depression has been shown to be correlated with induced tolerance against otherwise injurious intervals of cerebral ischemia in the rat.⁶ The extracellular signal-regulated kinase (ERK) signaling pathways are primary mediators of axonal growth and neuronal survival.^{7,8} ERK 1/2 has been reported to be activated by BDNF in cortical,⁹ hippocampal,¹⁰ and motor neurons.¹¹ On the basis of these studies, ERK 1/2 is considered to be a useful marker and survival intracellular signal transduction of neurotrophic activation by BDNF.

Recently it has been shown that activated neutrophils play a central role in the development of ischemia-reperfusion-induced central nerve injury.¹² Activated neutrophils may produce cerebral damage by means of free radicals, elastase, and cytokines. Furthermore, it has been reported that inhibition of neutrophil elastase decreases cytokine-induced neutrophil chemoattractants and that neutrophil elastase stimulates intercellular adhesion molecule 1 expression.¹³ ONO-5046, N-(2-[4-{2,2-dimethylpropionyloxy}phenylsulfonylamino]benzoyl)aminoacetate tetrahydrate, is a potent, specific, and intravenously active neutrophil elastase inhibitor.¹⁴ ONO-5046 can attenuate tissue or organ injury in various pathologic conditions by specifically inhibiting neutrophil elastase.¹⁵ However, the possibility that ONO-5046 may be effective for protecting motor neurons in the spinal cord from ischemic injury has not yet been examined. In this study, we investigated the protective effects of ONO-5046 after transient spinal cord ischemia and found that it reduced the apoptotic signaling of caspase3, prolonged the presence of the cell survival signals of phosphorylated ERK (p-ERK) and BDNF, and protected spinal cord motor neurons from ischemic injury.

Materials and Methods**Materials**

N-(2-[4-{2,2-dimethylpropionyloxy}phenylsulfonylamino]benzoyl)aminoacetate tetrahydrate (ONO-5046) was kindly provided by Ono Pharmaceutical Company (Osaka, Japan).

Animal Models

Thirty-three male domesticated white rabbits (Kitayama Rabess Company, Nagano, Japan), weighing 2.5 to 3 kg, were divided into three groups: sham operation control group (sham group), transient ischemia and treatment with vehicle group (group I), and transient ischemia and treatment with ONO-5046 group (10 mg/[kg · h], group O). All rabbits were allowed free access to food and water before and after the procedure, and were treated in accordance with the Declaration of Helsinki and the "Guide for the Care and Use of Laboratory Animals" (<http://www.nap.edu/catalog/5140.html>). The experimental and animal care protocols were approved by the Animal Care Committee of the Osaka University School of Medicine.

Anesthesia was induced by intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane in oxygen by inhalation. A 5F pediatric balloon-tipped catheter (model 405; Braun, Melsungen, Germany) was inserted through the right femoral artery and advanced 15 cm forward into the abdominal aorta. Preliminary experiments had already confirmed that the balloon in the distal end of the catheter should be positioned 0.5 to 1.5 cm distal to the left renal artery.¹⁶ The catheter was immediately removed without injection or balloon inflation in the sham group. In groups I and O, spinal cord ischemia was achieved by inflating the balloon to obstruct blood flow to the spinal cord. Our previous experiments confirmed that 15 minutes of transient spinal cord ischemia was sufficient for selective and delayed motor neuron death.¹⁷ Saline solution (vehicle, 1 mL/kg body weight) or ONO-5046 (5 mg/mL in saline vehicle) was administered intravenously from the induction of ischemia until 60 minutes of reperfusion. Immediately after death, the spinal cord was quickly removed with the plunger of a 1-mL syringe.¹⁸ All samples were frozen in powdered dry ice and stored at -80°C until use. The spinal cords were then cut transversely at approximately the L2 or L3 level and mounted on glass slides.

Neurologic Assessment

Neurologic function was assessed before the rabbits were killed at 7 days after reperfusion (n = 5 for each group). The rabbits were classified according to a 5-point scale devised by Johnson and colleagues¹⁹ as follows: 0, hind-limb paralysis; 1, severe paraparesis; 2, functional movement, no hop; 3, ataxia, uncoordinated hop; 4, minimal ataxia; and 5, normal function. Two individuals without knowledge of the treatment independently graded the neurologic function. The Mann-Whitney *U* test was used to compare the neurologic scores and cell numbers.

Histologic Study

Sections taken at 7 days after reperfusion in all groups were stained with hematoxylin-eosin and examined under light microscopy. An observer who was unaware of the animal group or neurologic outcome examined each slide. With hematoxylin-eosin, the cells were considered dead if the cytoplasm was diffusely

TABLE 1. Neurologic functional scores on day 7 after the procedure

Animal	Sham	Group I	Group O
1	5	3	5
2	5	2	5
3	5	2	5
4	5	2	3
5	5	4	4
Mean \pm SD	5	2.6 \pm 0.90	4.4 \pm 0.90*

* $P = .013$ versus group I.

eosinophilic and viable if the cells showed basophilic stripping (contained Nissl substance). The Mann-Whitney U test was used to compare the neurological scores and cell numbers.

BDNF, p-ERK, and Caspase3 Immunocytochemical Examination

We also performed an immunohistochemical study to investigate changes in the expressions of BDNF, p-ERK, and caspase3 at 8 hours, 1 day, and 2 days after reperfusion in groups I and O and at 2 days in the sham group ($n = 3$ each). Spinal cord sections were rinsed in 0.1 mol/L phosphate-buffered saline solution for 20 minutes and blocked in 2% normal horse serum for 2 hours at room temperature. Next, they were incubated with a primary antibody diluted 1:200 in 10% normal horse or rabbit serum and 0.3% Triton X-100 (The Dow Chemical Company, Midland, Mich) for 20 hours at 4°C. The primary antibodies used were as follows: sheep polyclonal anti-BDNF antibody (AB1513p; CHEMICON International Inc, Temecula, Calif), mouse polyclonal anti-ERK phosphospecific (SC-7383; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and mouse monoclonal anticaspase3 p20 (SC-1226; Santa Cruz Biotechnology) antibodies. After quenching of the endogenous peroxidase activity by incubation in 0.3% hydrogen peroxide and 10% methanol for 20 minutes, the slides were washed in phosphate-buffered saline solution and incubated for 3 hours with biotinylated anti-sheep IgG (PK-6106; Vector Laboratories, Inc, Burlingame, Calif), biotinylated anti-mouse IgG (PK-6102; Vector Laboratories), or biotinylated anti-goat IgG (PK-6105; Vector Laboratories) diluted 1:200 in phosphate-buffered saline solution containing 0.018% normal horse or rabbit serum. Next, the slides were incubated with avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories), followed by color development of the positive staining with 3,3'-diamino-benzidine and hydrogen peroxide solution and counterstaining of the cytoplasm with hematoxylin-eosin. Another set of sections were stained in a similar way but without the primary antibody to ascertain the specific binding of each antibody to its target protein.

Results

Neurologic Assessment

The neurologic results are summarized in Table 1. All the rabbits survived until the end of the observation period. All sham-operated control animals ($n = 5$) demonstrated normal neurologic function (score of 5). Until 2 days after

TABLE 2. Number of large motor neurons in the ventral gray matter on day 7 after the procedure

Animal group	No. of cells
Sham	19.0 \pm 1.0
Group I	7.8 \pm 3.1
Group O	16.8 \pm 3.4*

* $P = .0028$ versus group I.

reperfusion, neurologic function was preserved, and there was no significant difference between groups I and O (data not shown). Almost all the rabbits in group I demonstrated severe paraparesis by 7 days ($n = 5$). In group O, the deterioration of neurologic function was eased significantly relative to that in group I ($P = .013$).

Histologic Study

The numbers of intact motor neurons are summarized in Table 2. In sham-operated control animals, the spinal cord was intact with many large motor neurons in the anterior horn (Figure 1, A). Seven days after blood flow restoration in group I, however, about 60% of the motor neurons in the spinal cord were damaged (Figure 1, B). Seven days after reperfusion in group O (Figure 1, D), there was less evidence of damaged motor neurons. The number of intact motor neurons in group O was significantly larger than that in group I ($P = .0028$; Table 2).

Immunohistochemical Study

Table 3 shows a summary of the immunohistochemical analysis for each group. The spinal cords of sham-operated animals showed slight immunoreactivity for BDNF (Figure 2, A) but did not contain p-ERK (Figure 3, A) or caspase3 (Figure 4, A) immunoreactivity. After 15 minutes of transient ischemia, BDNF (Figure 2, B), p-ERK (Figure 3, B), and caspase3 (Figure 4, B) were induced in motor neurons after 8 hours of reperfusion but had almost disappeared at 1 day (Figure 2, C, Figure 3, C, and Figure 4, C, respectively). In group O, immunoreactivity for caspase3 was not observed (Figure 4, E, F, and G). Relative to group I, motor neurons showed prolonged immunoreactivity for BDNF (Figure 2, E and F) and p-ERK. (Figure 3, E and F) at 8 hours and 1 day after reperfusion and then started to weaken at 2 days (Figure 2, G, Figure 3, G).

Discussion

Activated neutrophils have been reported to contribute to ischemia-reperfusion injury in the brain and spinal cord.¹² Activated neutrophils may forward tissue damage through free radicals, elastase, and cytokines. Neutrophil elastase is an enzyme capable of damaging endothelial cells.²⁰ It also degrades extracellular matrix components, including elastin, fibronectin, proteoglycans, and collagen. The extracellular

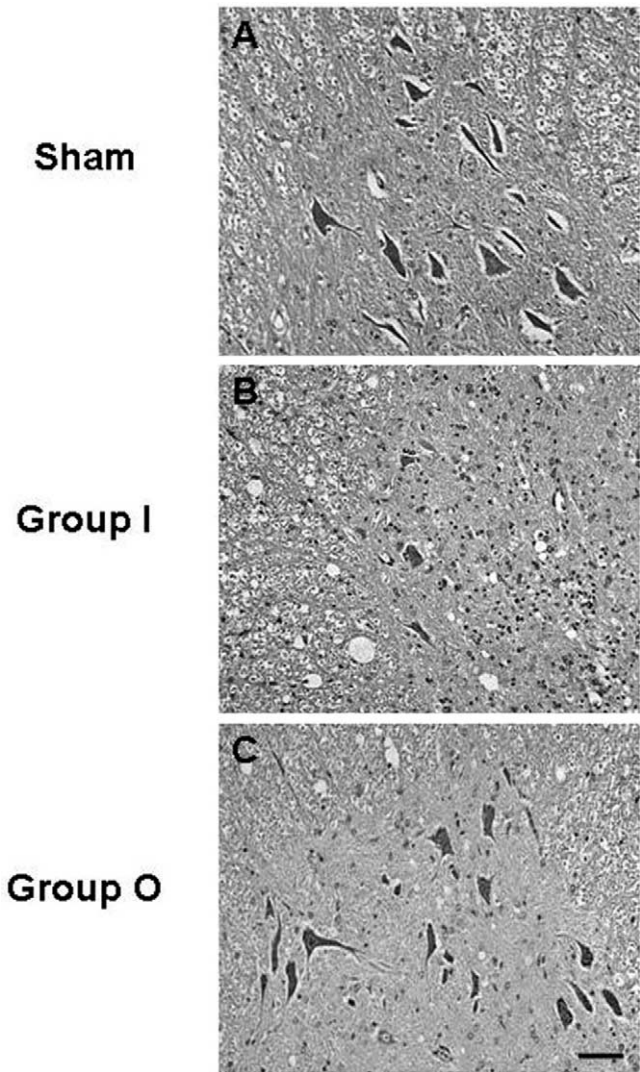


Figure 1. Histologic findings of spinal cord 7 days after 15 minutes of ischemia (hematoxylin-eosin staining). **A,** Sham-operated control spinal cord is intact. **B,** In spinal cord of group I (15 minutes of ischemia), about 60% of motor neurons are lost. **C,** In group O, there is less evidence of damaged motor neurons, and no glial changes are found. Bar represents 200 μ m.

matrix components of the endothelial basement membrane have a key function as a vascular barrier that limits fluid and solute exchange and may also restrict granulocyte extravasation.²¹ Neutrophil elastase increases vascular permeability and damages endothelial integrity.²² The endothelial damage also induces microcirculatory disturbance, leading to tissue ischemia and cellular damage.²¹

ONO-5046 is a new, specific, and reversible inhibitor of human neutrophil elastase that has shown beneficial effects in various tissue injuries.^{15,20} ONO-5046 has been reported to prevent neutrophil accumulation and reduce cerebral

TABLE 3. Summary of immunohistochemical analysis for each group (n = 3)

	8 h	1 d	2 d
BDNF			
Sham			$\pm \pm \pm$
Group I	$++ +$	$\pm \pm \pm$	$\pm \pm \pm$
Group O	$++ +$	$2+ 2+ 2+$	$++ \pm$
P-ERK			
Sham			!!!
Group I	$2+ 2+ 2+$	$\pm !!$!!!
Group O	$2+ 2+ 2+$	$2+ 2+ 2+$	$++ \pm$
Caspase3			
Sham			!!!
Group I	$2+ 2+ +$	$\pm \pm !$!!!
Group O	!!!	!!!	!!!

!, Negative; \pm , subtly positive reaction; +, positive reaction; 2+, strongly enhanced reaction.

ischemic damage in middle cerebral artery occlusion in a rat model.²³ However, the effects of this elastase inhibitor on neurologic function and intracellular signal transduction after transient ischemia in the spinal cord and brain have been unclear. We therefore investigated whether ONO-5046 had a preventive effect in the anterior horn of the spinal cord after transient ischemia. Treatment with ONO-5046 resulted in protection against paraplegia and demonstrated a protective effect on motor neurons. It also prevented the induction of caspase3 (CPP32) and prolonged the expressions of BDNF and p-ERK in motor neurons.

Caspase3 is a member of the interleukin-converting enzyme-like proteases, which are related to mammalian apoptosis and inflammation.²⁴ Our previous study demonstrated that an increase in the immunoreactivity of the apoptosis-inducing protein caspase3 occurred in the motor neuron cells of the spinal cord after 15 minutes of transient ischemia and that the peak of caspase3 induction preceded DNA fragmentation in the spinal cord after the ischemic insult.⁴ These findings suggest that overexpression of caspase3 may play an important role in the induction of DNA fragmentation in the spinal cord. The expression of caspase3 preceded the appearance of neuronal damage and may therefore have been involved in the activation of apoptosis.

BDNF is one of the neurotrophic factors and acts through specific receptors on neurons to effect biologic activities such as neuronal survival, stimulation of neurite outgrowth, and activation of neurotransmitter synthesis and release. The action of BDNF on motor neurons has been extensively studied in injury models and spontaneous motor nerve degeneration models. An active and potentially protective role for BDNF in inducing higher resistance of cerebral and spinal neurons against ischemia is further supported by the observation that intracerebroventricular BDNF infusion

CSP

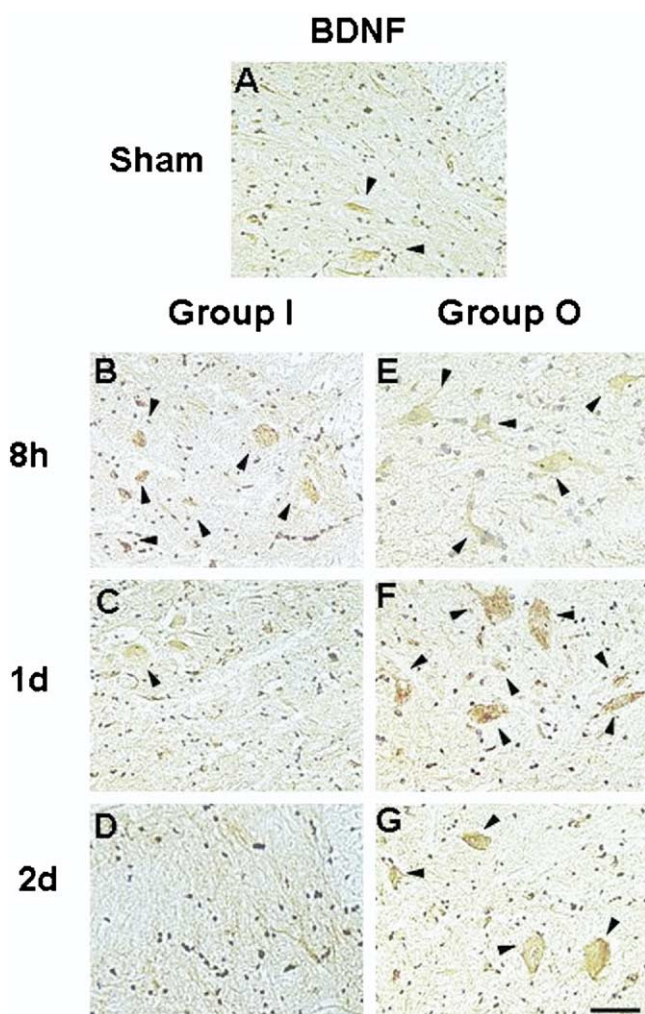


Figure 2. Immunohistochemical staining for BDNF in sham-operated control spinal cord (A) and in spinal cords at 8 hours (B), 1 day (C), and 2 days (D) in group I and at 8 hours (E), 1 day (F) and 2 days (G) in group O. Prolonged BDNF immunoreactivity is present in motor neurons of group O (black arrows). Bar represents 400 μ m.

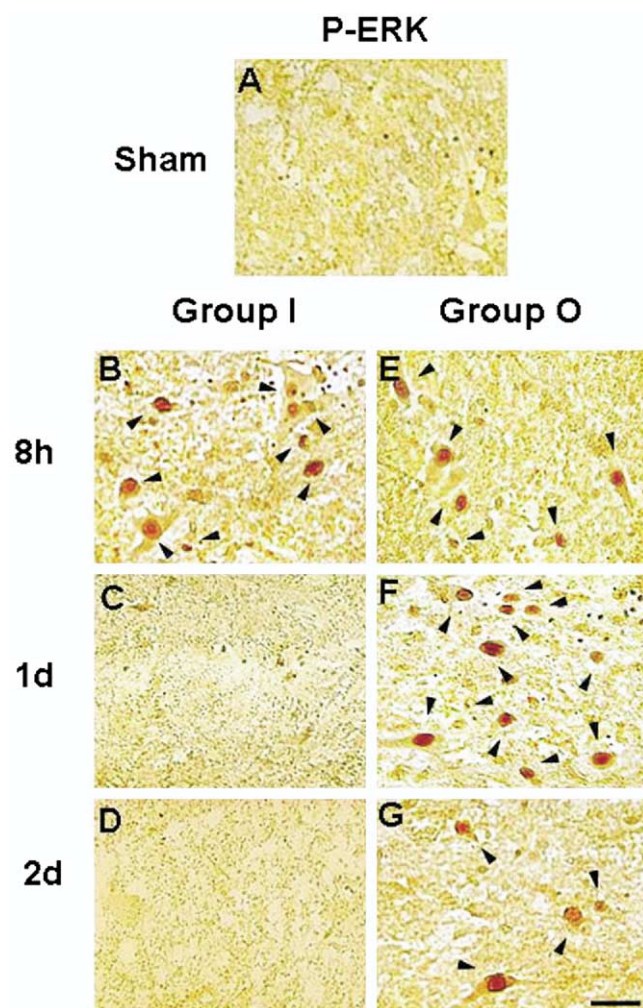


Figure 3. Immunohistochemical staining for p-ERK in sham-operated control spinal cord (A) and in spinal cords at 8 hours (B), 1 day (C), and 2 days (D) in group I and at 8 hours (E), 1 day (F), and 2 days (G) in group O. Prolonged p-ERK immunoreactivity is present in nuclei of motor neurons in group O (black arrows). Bar represents 400 μ m.

provides significant protection to CA1 hippocampal neurons after global cerebral ischemia in the rat.²⁵

The ERKs, downstream signal components of BDNF, regulate a diverse array of functions, such as cell growth and proliferation, differentiation, and apoptosis.²⁶ Phosphorylation is required for full enzymatic activation. The significance of a p-ERK increase for cell survival is somewhat uncertain, because previous studies have suggested both antiapoptotic and proapoptotic roles for p-ERK 1/2.^{26,27} In hypoxic neuronal injury with primary cultures from the murine cerebral cortex, the mitogen-activated protein kinase/ERK kinase (MEK) 1/2 inhibitor PD98059 increased neuronal death in hypoxic cultures, suggesting that MEK

1/2 promotes neuronal survival.²⁸ Moreover, PD98059 also reduced phosphoinactivation of Bad in hypoxic cultures, suggesting that a cell-survival program involving phosphoactivation of MEK 1/2 and its downstream ERK 1/2 and inactivation of Bad is mobilized in hypoxic neurons and may help to regulate neuronal fate after hypoxic-ischemic injury.

In the neonatal brain, BDNF is markedly neuroprotective against neonatal hypoxic-ischemic brain injury in vivo.²⁹ Intracerebroventricular administration of BDNF to rats on postnatal day 7 resulted in phosphorylation of ERK 1/2. Pharmacologic inhibition of ERK inhibited the ability of BDNF to block hypoxic-ischemic caspase3 activation and tissue loss. These results suggest that p-ERK may be in-

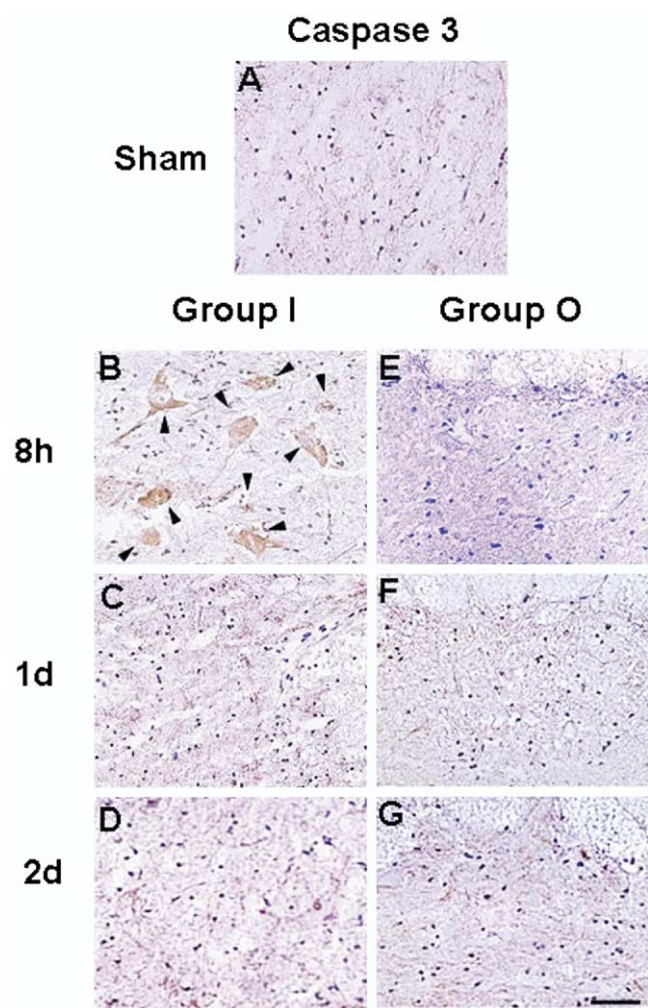


Figure 4. Immunohistochemical staining for caspase3 in sham-operated control spinal cord (A) and in spinal cords at 8 hours (B), 1 day (C), and 2 days (D) in group I and at 8 hours (E), 1 day (F), and 2 days (G) in group O. Density of caspase3 immunoreactivity in group O is reduced relative to that in group I (black arrows). Bar represents 400 μ m.

involved in cell survival signaling in central nerves after ischemia. In our study, BDNF and p-ERK proteins were expressed in an early stage of reperfusion in motor neuron cells of the ventral gray matter. The induction reached a maximum at 8 hours and almost disappeared at 1 day. It has been demonstrated that there is significant upregulation of BDNF in peri-ischemic regions after a noninjurious interval of middle cerebral artery occlusion in the rat.⁵ We have previously reported the induction of glial cell line-derived neurotrophic factor and *c-ret* proto-oncogene receptor tyrosine kinase after transient spinal cord ischemia in the rabbit.¹⁶ In a rat model, a transient, noninjurious interval of spinal ischemia led to significant increases in spinal glial

cell line-derived neurotrophic factor and BDNF expressions.³⁰ A variety of pathologic insults, including ischemia, have been reported to upregulate several growth factors, and these can play important roles in modulating the survival of brain and spinal cord neurons. The prolonged expressions of BDNF and its important signaling component p-ERK therefore may be among the factors responsible for easing spinal cord ischemic damage. However, the exact mechanism of the prolonged expression of survival cell signals after ONO-5046 treatment remains unclear. We previously demonstrated that the expression of heat shock protein the spinal cord was prolonged for local hypothermic ischemia relative to normothermic ischemia.¹⁷ One possible hypothesis for this is as follows: transient ischemia induces the stress response of motor neurons, such as induction of some growth factors and stress response proteins, and motor neurons less damaged as a result of possibly beneficial treatments (such as hypothermia or medicines) may have the ability to prolong the production of these proteins relative to neurons without such treatments.

The mechanism of neutrophil-mediated spinal cord ischemia injury is not fully understood, especially with regard to intracellular cell signal transduction. Inhibition of neutrophil elastase by ONO-5046 may ease ischemic motor damage by prolonging the expression of survival signal transduction molecules, including BDNF and p-ERK, and suppressing the expression of the death signal transduction molecule caspase3. A balance between cell survival signals (such as BDNF and p-ERK) and death signals (such as activated caspase3) may be important for determining the cell fate of survival or death under both normal and pathologic conditions. Neutrophils may affect the survival and death-promoting events in spinal cord ischemic injury.

This study has revealed that ONO-5046 can protect the motor neurons of the spinal cord from ischemic injury. Inhibition of neutrophil elastase reduces the induction of caspase3 and prolongs the expression of BDNF and p-ERK. In the near future, ONO-5046 should therefore be a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury.

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